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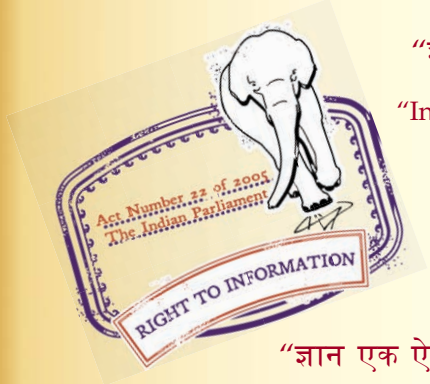
IS 4684 (1975): Edible Groundnut Flour (Expeller Pressed)
[FAD 16: Foodgrains, Starches and Ready to Eat Foods]



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(First Revision)

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(Reaffirmed 1983)

Indian Standard

SPECIFICATION FOR EDIBLE GROUNDNUT FLOUR (EXPELLER PRESSED)

(First Revision)

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AMENDMENT NO. 1 FEBRUARY 1986

TO

IS:4684-1975 SPECIFICATION FOR EDIBLE GROUNDNUT FLOUR (EXPELLER PRESSED)

(First Revision)

(*Page 4, clause 2.2.1, line 3*) - Substitute '11 and 12 respectively of IS:7874(Part 1)-1975‡' *for* '11 of IS:1714-1960‡ and in Appendix A of IS:1712-1972§'.

(*Page 4, foot-notes with* '‡' *and* '§' *marks*) - Substitute the following for the existing foot-notes:

'‡Method of sampling and test for animal feeds and feeding stuffs : Part 1 General methods.'

[*Page 5, Table 1, Sl No.* (vi), *col* (3)] - Substitute '6.0' *for* '4.0'.

(AFDC 37)

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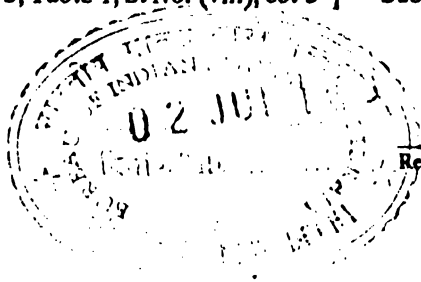
AMENDMENT NO. 2 JULY 1999
TO
IS 4684 : 1975 SPECIFICATION FOR EDIBLE GROUNDNUT FLOUR (EXPELLER PRESSED)

(First Revision)

[*Page* 5, *Table* 1, *Sl No.* (viii), *col* 3] — Substitute '30' *for* '60'.

(FAD 15)



Reprography Unit, BIS, New Delhi, India

Indian Standard

SPECIFICATION FOR EDIBLE GROUNDNUT FLOUR (EXPELLER PRESSED)

(First Revision)

0. FOREWORD

0.1 This Indian Standard (First Revision) was adopted by the Indian Standards Institution on 30 October 1975, after the draft finalized by the Nutrition Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Edible groundnut flour is well recognized as a rich source of dietary protein. Its properties, such as taste, odour and colour have led to its use in many food products like biscuits, *BALAHAR* and other snack foods.

0.3 This Indian Standard was first published in 1968. Consequent upon improvement in the quality of the edible groundnut flour produced commercially, it was felt that the standard required to be modified. Accordingly, this revision is being issued. In this revision the requirement for available lysine has been excluded, limit for aflatoxin reduced and description of raw material preparation elaborated.

0.4 One of the toxicants which is usually present in edible groundnut protein products is aflatoxin, produced by a fungus (*Aspergillus flavus*). It is now well-established that aflatoxin is harmful to human beings when ingested even in minute quantities. It, therefore, becomes imperative that the edible flour is produced under strictly controlled hygienic conditions. Under optimum conditions of growth, harvesting, drying, the toxin content can be almost negligible. At the same time several methods have been developed for detoxification of the aflatoxin present in edible groundnut flour, by treatment with chemicals like ammonia, hydrogen peroxide and certain oxidizing agents. Simultaneously, these treatments bring about a lowering of the nutritive value of the edible groundnut protein products through destruction of the sulphur amino acids. Therefore, safe methods of making edible groundnut protein products of good nutritive value and low aflatoxin content are either by treating groundnut pods in the field to avoid fungal contamination, or by manually removing fungus-affected kernels before processing the remainder, or by using groundnut naturally resistant to fungus. At present, manual removal of fungus-affected kernel is adopted for

commercial production of edible groundnut flour, and products with an aflatoxin content well below the prescribed limit are regularly obtained.

0.5 A separate Indian Standard (IS : 4875-1975*) for solvent extracted (variety of groundnut flour) has also been formulated.

0.6 This standard has been formulated in close collaboration with the Protein Foods and Nutrition Development Association of India. While formulating this standard due consideration has been given to relevant Rules issued by the Government of India under the Prevention of Food Adulteration Act, 1954. This standard is, however, subject to the restrictions imposed under that Act, wherever applicable.

0.7 For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of test or analysis, shall be rounded off in accordance with IS : 2-1960†. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

1. SCOPE

1.1 This standard prescribes the requirements, and the methods of sampling and test for edible groundnut flour (expeller pressed).

2. REQUIREMENTS

2.1 Raw Material — Expeller pressed edible groundnut flour shall be made by expelling in a screw press fresh, clean degermed groundnut kernels which have been decuticled after mild roasting. In order to reduce the proportion of immature, shrivelled and mouldy kernels which could carry high levels of aflatoxin, the kernels shall be selected either by visual inspection, inspection under ultraviolet light, electronic sorting or by other means. The kernels shall be free from insect or fungal infestation.

2.2 Description — Expeller pressed edible groundnut flour shall be whitish to light brown in colour, uniform in composition and shall be free from insect or fungal infestation, objectionable odour and rancid taste. It shall not contain added flavouring and colouring agents or any other extraneous matter.

2.2.1 Expeller pressed edible groundnut flour shall be free from castor husk or *MAHUA* oilcake when tested according to the methods prescribed in **11** of IS : 1714-1960‡ and in Appendix A of IS : 1712-1972§, respectively.

*Specification for edible groundnut flour (solvent extracted) (*first revision*).
†Rules for rounding off numerical values (*revised*).
‡Methods of sampling and test for oilcakes as livestock feed.
§Specification for cottonseed oilcake as livestock feed (*first revision*).

2.3 Particle Size — Unless otherwise specified the material shall be of such fineness that, when tested by the method prescribed in Appendix A, not more than 5 percent by mass shall be retained on a 710-micron IS Sieve (*see* IS : 460-1962*) and not more than 20 percent by mass shall be retained on a 500-micron IS Sieve.

2.4 Premises — Place where manufacture, packing and storage of the material is done, and the eqipment empolyed, shall be maintained under hygienic conditions (*see* IS : 2491-1972†).

2.5 Edible groundnut flour (expeller pressed) shall also comply with the requirements given in Table 1.

TABLE 1 REQUIREMENTS FOR EDIBLE GROUNDNUT FLOUR (EXPELLER PRESSED)

Sl No.	Characteristic	Requirement	Method of Test (Ref to Appendix)
(1)	(2)	(3)	(4)
i)	Moisture, percent by mass, *Max*	9·0	B
ii)	Protein (N × 6·25) (on dry basis), percent by mass, *Min*	45·0	C
iii)	Total ash (on dry basis), percent by mass, *Max*	4·5	D
iv)	Acid-insoluble ash (on dry basis), percent by mass, *Max*	0·35	E
v)	Fat (on dry basis), percent by mass, *Max*	9·0	F
vi)	Acid value of extracted fat, *Max*	4·0	G
vii)	Crude fibre (on dry basis), percent by mass, *Max*	5·0	H
viii)	Aflatoxin, μ g/kg, *Max*	60	J

2.6 Bacteriological Requirements — The edible groundnut flour (expeller pressed) shall be tested periodically to comply with the requirements given in Table 2.

3. PACKING AND MARKING

3.1 Packing — The material shall be packed in polyethylene or polyethylene-lined jute bags, or in clean tinplate containers. When packed in bags the mouth of each bag shall be either machine- or hand-stitched. If hand-stitched, the mouth shall be rolled over and stitched. Stitches shall be in two cross-rows with at least 14 stitches in each row.

*Specification for test sieves (*revised*).
†Code for hygienic conditions for food processing units (*first revision*).

3.2 Marking — The following particulars shall be marked or labelled on each container :

a) Name of the material,

b) Name and address of the manufacturer,

c) Batch or code number,

d) Net mass, and

e) Date of manufacture.

TABLE 2 BACTERIOLOGICAL REQUIREMENTS FOR EDIBLE GROUNDNUT FLOUR (EXPELLER PRESSED)

(*Clause* 2.6)

Sl No.	Characteristic	Requirement	Method of Test, Ref to
(1)	(2)	(3)	(4)
i)	Total bacterial count per g, *Max*	50 000	IS : 5402-1969*
ii)	Coliform bacterial count per g, *Max*	10	IS : 5401-1969†
iii)	*Salmonella* bacteria	Nil	IS : 5887-1970‡

*Method for standard plate count of bacteria in foodstuffs.

†Methods for detection and estimation of coliform bacteria in foodstuffs.

‡Methods for detection of bacteria responsible for food poisoning and food-borne diseases.

3.3 BIS Certification Marking

The product may also be marked with Standard Mark.

3.3.1 The use of the Standard Mark is governed by the provisions of the Bureau of Indian Standards Act, 1986 and the Rules and Regulations made thereunder. The details of conditions under which the licence for the use of Standard Mark may be granted to manufacturers or producers may be obtained from the Bureau of Indian Standards.

4. SAMPLING

4.1 Representative samples of the material shall be drawn and tested for conformity to this standard as prescribed in IS : 5315-1969*.

5. TESTS

5.1 Tests shall be carried out as prescribed in **2.2** and **2.3**, and Tables 1 and 2.

*Methods of sampling for milled cereals and pulses products.

5.2 Quality of Reagents — Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1960†) shall be employed in tests.

NOTE — ' Pure chemicals ' shall mean chemicals that do not contain impurities which affect the test results.

APPENDIX A

(*Clause* 2.3)

DETERMINATION OF PARTICLE SIZE

A-1. TEST SIEVES

A-1.1 Make a nest of two test sieves, the upper being 710-micron IS Sieve and the lower 500-micron IS Sieve provided with a cover and receiver.

NOTE — In case the prescribed IS Sieves are not available, the following corresponding sieves, which have their apertures within the limits specified for the prescribed one, may be used :

IS Sieve	*Corresponding Sieve*
710-micron	BS Test Sieve 22, ASTM Sieve 25 or Tyler Test Sieve 24
500-micron	BS Test Sieve 30, ASTM Sieve 35 or Tyler Test Sieve 32

A-2. PROCEDURE

A-2.1 Weigh accurately about 100 g of the material and transfer to the upper sieve. Fit the upper sieve with the cover, place the nest of sieves in a suitable mechanical sieve shaker and sieve the material continuously for five minutes. Stop sieving, remove the nest of sieves and transfer the residue on each sieve separately to two tared weighing dishes using a brush. Weigh each dish.

A-3. CALCULATION

A-3.1 Calculate the percentage of the material retained on the respective sieves as follows :

a) Material retained on 710-micron

$$\text{IS Sieve, percent by mass} = \frac{100\ M_1}{M}$$

where

M_1 = mass in g of the material retained on 710-micron IS Sieve, and

M = mass in g of the material taken for the test.

†Specification for water, distilled quality (*revised*)

b) Material retained on 500-micron

IS Sieve, percent by mass $= \frac{100\ M_2}{M}$

where

M_2 = mass in g of the material retained on 500-micron IS Sieve, and

M = mass in g of the material taken for the test.

APPENDIX B

[*Table* 1, *Item* (i)]

DETERMINATION OF MOISTURE

B-1. PROCEDURE

B-1.1 Weigh accurately about 10 g of the material in a dish made of porcelain, silica or platinum, previously dried in an air-oven and weighed. Place the dish in an air-oven maintained at 105 ± 2°C for five hours. Cool the dish in a desiccator and weigh with the lid on. Heat again at 105 ± 2°C in the air-oven for 30 minutes. Cool the dish in the desiccator and weigh. Repeat this process of heating for 30 minutes, cooling and weighing till the difference in mass between two successive weighings is less than one milligram. Note the lowest mass.

NOTE — Preserve the dish containing this dried material for determination of total ash (*see* **D-1.1**).

B-2. CALCULATION

B-2.1 Calculate the moisture as follows :

Moisture, percent by mass $= \frac{100\ (M_1 - M_2)}{M_1 - M}$

where

M_1 = mass in g of the dish with the material before drying,

M_2 = mass in g of the dish with the material after drying, and

M = mass in g of the empty dish.

APPENDIX C

[*Table* 1, *Item* (ii)]

DETERMINATION OF PROTEIN

C-1. APPARATUS

C-1.1 A recommended apparatus, as assembled, is shown in Fig. 1.

C-1.1.1 *Description* — The assembly consists of a round-bottom flask *A* of 1 000-ml capacity fitted with a rubber stopper through which passes one end

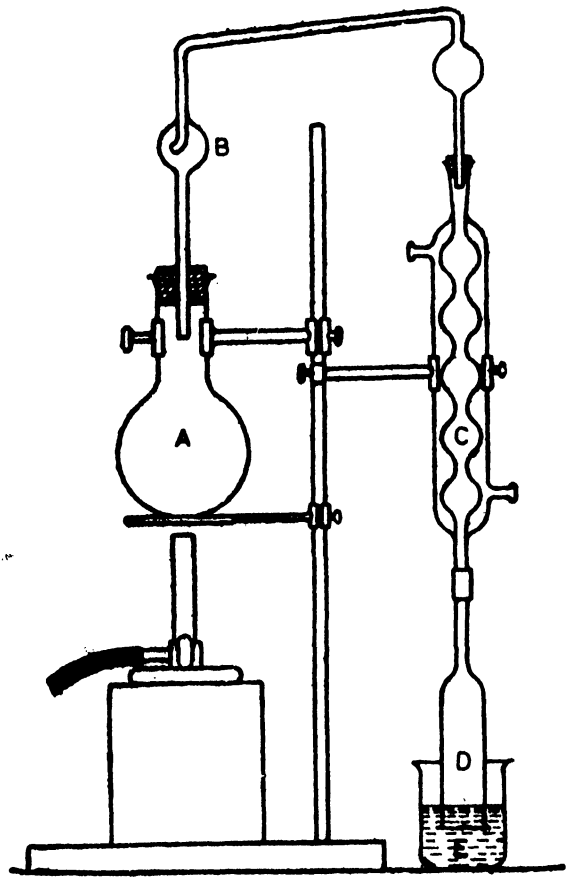


FIG. 1 APPARATUS FOR DETERMINATION OF PROTEIN

of the connecting bulb tube *B*. The other end of the bulb tube *B* is connected to the condenser *C* which is attached by means of a rubber tube to a dip tube *D* which dips into a known quantity of standard sulphuric acid contained in a beaker *E* of 250-ml capacity.

C-1.2 Kjeldahl Flask — of capacity 500 ml.

C-2. REAGENTS

C-2.1 Anhydrous Sodium Sulphate

C-2.2 Copper Sulphate

C-2.3 Concentrated Sulphuric Acid — sp gr 1·84.

C-2.4 Sodium Hydroxide Solution — Dissolve about 225 g of sodium hydroxide in 500 ml of water.

C-2.5 Standard Sulphuric Acid — 0·1 N.

C-2.6 Methyl Red Indicator Solution — Dissolve 1 g of methyl red in 200 ml of rectified spirit (95 percent by volume).

C-2.7 Standard Sodium Hydroxide Solution — 0·1 N.

C-3. PROCEDURE

C-3.1 Transfer carefully about 0·25 g of the material, accurately weighed, to the Kjeldahl flask, taking precaution to see that particles of the material do not stick on to the neck of the flask. Add about 10 g of anhydrous sodium sulphate, about 0·2 to 0·3 g of copper sulphate and 20 ml of concentrated sulphuric acid. Place the flask in an inclined position. Heat below the boiling point of the acid until frothing ceases. Increase the heat until the acid boils vigorously and digest for 30 minutes after the mixture becomes clear and pale green or colourless. Cool the contents of the flask. Transfer it quantitatively to the round-bottom flask with water, the total quantity of water used being about 200 ml. Add with shaking a few pieces of pumice stone to prevent bumping.

Add about 50 ml of sodium hydroxide solution (which is sufficient to make the solution alkaline) carefully through the side of the flask so that it does not mix at once with the acid solution but forms a layer below the acid layer. Assemble the apparatus as shown in Fig. 1, taking care that the dip tube extends below the surface of known quantity of standard sulphuric acid contained in the beaker. Mix the contents of the flask by shaking and distil until all ammonia has passed over into the standard sulphuric acid. Shut off the burner and immediately detach the flask from the condenser. Rinse the condenser thoroughly with water into the beaker. Wash the dip tube carefully so that all traces of the condensate are transferred to the beaker,

When all the washings have drained into the beaker, add two or three drops of methyl red indicator solution and titrate with the standard sodium hydroxide solution.

C-3.2 Carry out a blank determination using all reagents in the same quantities but without the material to be tested.

C-4. CALCULATION

C-4.1 Calculate protein from the following formula :

$$\text{Protein (on dry basis), percent by mass} = \frac{875\,(B - A)\,N}{M\,(100 - m)}$$

where

B = volume in ml of the standard sodium hydroxide solution used to neutralize the acid in the blank determination;

A = volume in ml of the standard sodium hydroxide solution used to neutralize the excess of acid in the test with the material;

N = normality of the standard sodium hydroxide solution;

M = mass in g of the material taken for the test; and

m = moisture, percent by mass, in the material (*see* **B-2.1**).

APPENDIX D

[*Table* 1, *Item* (iii)]

DETERMINATION OF TOTAL ASH

D-1. PROCEDURE

D-1.1 Ignite the dried material in the dish (**B-1.1**) with the flame of a suitable burner for about one hour. Complete the ignition by keeping in a muffle furnace at 550 to 600°C until grey ash results. Cool in a desiccator and weigh. Repeat the process of igniting, cooling and weighing at half-hour intervals until the difference in mass between two successive weighings is less than one milligram. Note the lowest mass.

NOTE — Preserve the dish containing this ash for the determination of acid insoluble ash (**E-2.1**).

D-2. CALCULATION

D-2.1 Calculate total ash as follows :

$$\text{Total ash (on dry basis), percent by mass} = \frac{100\,(M_2 - M)}{M_1 - M}$$

where

M_2 = mass in g of the dish with the ash,

M = mass in g of the empty dish, and

M_1 = mass in g of the dish with the dried material taken for the test (M_2 under **B-2.1**).

APPENDIX E

[*Table* 1, *Item* (iv)]

DETERMINATION OF ACID-INSOLUBLE ASH

E-1. REAGENT

E-1.1 Dilute Hydrochloric Acid — approximately 5 N, prepared from concentrated hydrochloric acid (*see* IS : 265-1962*).

E-2. PROCEDURE

E-2.1 Add 25 ml of dilute hydrochloric acid to the ash contained in the dish (**D-1.1**), cover with a watch-glass and heat on a water-bath for 10 minutes. Allow to cool and filter the contents of the dish through a Whatman filter paper No. 42 or its equivalent. Wash the filter paper with water until the washings are free from the acid. Return the filter and the residue to the dish. Keep it in an electric air-oven maintained at 135 ± 2°C for about 3 hours. Ignite in a muffle furnace at 550 to 600°C for one hour. Cool the dish in a desiccator and weigh. Repeat the process of igniting in the muffle furnance, cooling and weighing at half-hour intervals until the difference in mass between two successive weighings is less than one milligram. Note the lowest mass.

E-3. CALCULATION

E-3.1 Calculate the ash content from the following formula:

$$\text{Acid-insoluble ash (on dry basis), percent by mass} = \frac{100\ (M_2 - M)}{M_1 - M}$$

where

M_2 = mass in g of the dish with the acid-insoluble ash,

M = mass in g of the empty dish, and

M_1 = mass in g of the dish with the dried material (M_2 under **B-2.1**).

*Specification for hydrochloric acid (*revised*).

APPENDIX F

[*Table* 1, *Item* (v)]

DETERMINATION OF FAT

F-1. APPARATUS

F-1.1 Soxhlet Extraction Apparatus

F-2. SOLVENT

F-2.1 Ethyl Ether, or Petroleum Ether — distilling below 65°C.

F-3. PROCEDURE

F-3.1 Transfer about 10 g of the material accurately weighed to a suitable thimble and extract with the solvent in a Soxhlet extraction apparatus for about 16 hours. Dry the extract contained in the Soxhlet flask, whose empty mass has been previously determined at 95 to 100°C for 30 minutes. Cool in a desiccator and weigh. Continue drying and weighing alternately at 30-minute intervals until the loss in mass between two successive weighings is note more than one milligram. Record the lowest mass.

NOTE — Preserve the material in the thimble for estimation of crude fibre (*see* Appendix H).

F-4. CALCULATION

F-4.1 Calculate the fat as follows :

$$\text{Fat, percent by mass} = \frac{100\ (M_1 - M_2)}{M}$$

where

M_1 = mass in g of the Soxhlet flask with the extracted fat;

M_2 = mass in g of the empty Soxhlet flask, clean and dry; and

M = mass in g of the material taken for the test.

APPENDIX G

[*Table* 1, *Item* (vi)]

DETERMINATION OF ACID VALUE OF EXTRACTED FAT

G-1. APPARATUS

G-1.1 Soxhlet Fat Extraction Apparatus

G-2. REAGENTS

G-2.1 Petroleum Ether — distilling below 65°C,

G-2.2 Benzene-Alcohol-Phenolphthalein Solution — Add 0·4 g of phenolphthalein to one litre of benzene and one litre of ethyl alcohol to form 0·02 percent solution.

G-2.3 Standard Potassium Hydroxide Solution — 0·2 N, carbon dioxide-free.

G-2.4 Standard Potassium Permanganate Solution — 0·01 percent.

G-3. PROCEDURE

G-3.1 Extract 10·00 ± 0·01 g of the sample taken in a thimble with petroleum ether for about 16 hours in a Soxhlet extraction apparatus. Completely evaporate the solvent from extraction flask (weighed previously) on a steam-bath, cool and weigh the Soxhlet flask with the residue and from the difference of the mass find out the mass of fat obtained from the material. Dissolve the residue in the extraction flask with 50 ml of the benzene-alcohol-phenolphthalein solution. Titrate the dissolved extract with standard potassium hydroxide solution to distinct pink colour, or in the case of yellow solution to orange pink colour. If emulsion is formed during titration, dispel by adding second 50-ml portion of the benzene-alcohol-phenolphthalein solution. The end-point should match colour of the solution made by adding 2·5 ml of standard potassium permanganate solution to 50 ml of potassium dichromate solution of proper strength to match colour of original solution being titrated. (Add 0·5 percent potassium dichromate solution dropwise to 50 ml of water until the colour matches. Then add 2·5 ml of standard potassium permanganate solution.)

G-3.2 Make a blank titration on 50 ml of the benzene-alcohol-phenolphthalein solution and subtract this value from the titration value of the sample. If the additional 50-ml portion of the benzene-alcohol-phenolphthalein solution is added, double the blank titration.

G-4. CALCULATION

G-4.1 Calculate the acid value from the following formula :

$$\text{Acid value (as oleic acid)} = \frac{56{\cdot}4\ VN}{M}$$

where

V = volume in ml of standard potassium hydroxide solution used,

N = normality of standard potassium hydroxide solution, and

M = mass in g of the material taken for the test.

APPENDIX H

[*Table* 1, *Item* (vii)]

DETERMINATION OF CRUDE FIBRE

H-1. REAGENTS

H-1.1 Dilute Sulphuric Acid — 1·25 percent (*m/v*), accurately prepared.

H-1.2 Sodium Hydroxide Solution — 1·25 percent (*m/v*), accurately prepared.

H-1.3 Ethyl Alcohol — 95 percent by volume.

H-1.4 Petroleum Ether — distilling below 65°C.

H-2. PROCEDURE

H-2.1 Dry to constant mass about 5 g of the material on an electric oven at 105 ± 1°C. Weigh accurately about 2·5 g of the dried material on a thimble, extract it with petroleum ether free of fat on a Soxhlet apparatus and transfer it to a one-litre flask. Take 200 ml of dilute sulphuric acid in a beaker and bring to the boil. Transfer the whole of the boiling acid to the flask and immediately connect the flask with a water-cooled reflux condenser and heat, so that the contents of the flask begin to boil within one minute. Rotate the flask frequently, taking care to keep the material from remaining on the sides of the flask and out of contact with the acid. Continue boiling for exactly 30 minutes. Remove the flask and filter through fine linen (about 18 threads to a centimetre) held in a funnel, and wash with boiling water until the washings are no longer acid to litmus. Bring to the boil 200 ml of sodium hydroxide solution under a reflux condenser. Wash the residue on the linen into the flask with the boiling sodium hydroxide solution. Immediately connect the flask with the reflux condenser and boil for exactly 30 minutes. Remove the flask and immediately filter through the filtering cloth. Thoroughly wash the residue with boiling water, and transfer to a Gooch crucible prepared with a thin but compact layer of ignited asbestos. Wash the residue thoroughly first with hot water and then with about 15 ml of ethyl alcohol. Dry the Gooch crucible and contents at 105 ± 2°C in an air-oven to constant mass. Cool and weigh. Incinerate the contents of the Gooch crucible in an electric muffle furnance at 600 ± 2°C until all the carbonaceous matter is burnt. Cool the Gooch crucible containing the ash in a desiccator and weigh.

H-3. CALCULATION

H-3.1 Calculate the crude fibre as given below :

$$\text{Crude fibre (on dry basis), percent by mass} = \frac{100\,(M_1 - M_2)}{M}$$

where

M_1 = mass in g of the Gooch crucible and contents before ashing;

M_2 = mass in g of the Gooch crucible and contents after ashing; and

M = mass in g of the dried material taken for the test.

APPENDIX J

[*Table* 1, *Item* (viii)]

DETERMINATION OF AFLATOXIN

J-1. PRINCIPLE OF THE METHOD

J-1.1 The method involves direct extraction of a water-wetted sample by chloroform with the use of silica gel column for defatting and clean up for obtaining clean quantitative extracts of aflatoxin from 50 g of groundnut product samples for thin-layer chromatography. The low level of interference permits the determination and detection of as little as 1 μg of aflatoxin per kilogram of groundnut product sample.

J-2. APPARATUS

J-2.1 Disc Mill

J-2.2 Wrist-Action Shaker — having a capacity of 1 400 to 1 600 rev/min.

J-2.3 Chromatographic Column — of silica gel.

J-2.4 Funnel — having a diameter of 150 mm to fit fluted filter paper. Buchner funnel of 320 mm in diameter to fit Whatman No. 1 or equivalent filter paper may also be used.

J-2.5 Rotary Evaporator with Continuous Feed

J-2.6 Thin-Layer Chromatographic Apparatus

J-2.6.1 *Glass Plates* — 200 × 200 mm.

J-2.6.2 *Applicator*

J-2.6.3 *Mounting Board*

J-2.6.4 *Spotting Template*

J-2.6.5 *Microsyringe* — 10 µl.

J-2.6.6 *Desiccating Storage Cabinet*

J-2.6.7 *Storage Rack*

J-2.6.8 *Lone Wave 15-Watt Ultraviolet Lamp or Chromato-View Cabinet* — equipped with one or two 15-watt lamps.

J-3. REAGENTS

J-3.1 Silica Gel — for chromatography 0·15 to 0·2 mm.

J-3.2 Chloroform

J-3.3 Hexane

J-3.4 Diethyl Ether

J-3.5 Methyl Alcohol

J-3.6 Acetone

J-3.7 Sodium Sulphate (Anhydrous)

J-3.8 Boiling Chips

J-3.9 Aflatoxin Standard Solution — Prepare from pure crystalline aflatoxine solutions containing 0·5 µg of aflatoxin B_1 and 0·4 µg of aflatoxin G_1 per millilitre of chloroform. If pure crystalline aflatoxin is not available, use extracts of known aflatoxin content. Concentrations of 0·5 to 1·5 µg of B_1 and 0·3 to 1·0 µg of G_1 per millilitre are satisfactory. Keep exposure to light at minimum. Store at freezing temperature in glass stopper volumetric flask. Place in a closed jar containing a small amount of chloroform which should be replaced periodically. Chloroform in outside jar eliminates concentration of standard solution by evaporation and the jar prevents contamination with condensing water as standard solution is warm to room temperature each time it is used.

J-4. PROCEDURE

J-4.1 Preparation of the Silica Gel Plate — Weigh 30 g of silica gel into a 300-ml glass stoppered Erlenmeyer flask. Add 60 ml of distilled water. Shake vigorously for not more than a minute and pour into the applicator. Immediately coat five 200 × 200 mm glass plates with 0·25-mm thickness of silica gel suspension, and let the plates rest undisturbed until gelled. Dry the coated plates for at least 2 hours at 80°C and store in the desiccating cabinet with active desiccant until just before use.

J-4.2 Preparation of the Sample — Groundnut butters and groundnut meals need no preparation for extraction unless they contain large particles, in which case some type of milling operation should be used to reduce the

particle size. A hammer mill of rotary cutter is used for meals. Samples of raw and roasted groundnut should be ground to a paste before extraction. For this operation and for chunky-type groundnut butter, a disc-type mill is used.

J-4.3 Extraction — Weigh a 50-g sample (groundnut butter, groundnut meal, or finely ground groundnuts) into a 500-ml glass-stoppered Erlenmeyer flask. And 25 ml of distilled water and mix with a spatula until visually uniform. Add 250 ml of chloroform and tape the stopper in place. Shake for 30 minutes on a wrist-action shaker and filter through a fluted filter paper. Collect the first 50-ml portion of clear chloroform. This is the sample extract. Prepare the silica gel column by placing a ball of glass wool loosely in the bottom of a 22 × 300 mm chromatographic column and add 5 g of anhydrous sodium sulphate to give an even base for the silica gel. Add chloroform until the column is about two-thirds full; then add 10 g of silica gel (0·05 to 0·20 mm) with stirring to prevent air entrapment. Let silica gel settle and slowly add 15 g of anhydrous sodium sulphate. Draw off chloroform to the top of the silica gel. Add the sample extract to the column and elute at a flow rate of 10 to 20 ml per minute with 150 ml of hexane followed by 150 ml of anhydrous diethyl ether. Finally, elute the aflatoxin with 150 ml of methyl alcohol or chloroform (3/97, *v/v*). Collect this fraction from the time the methyl alcohol or chloroform was added until the flow stops in a vial, evaporate to near dryness and cap it.

J-4.3.1 The sample extract may now be prepared in the appropriate manner for quantitative assay.

J-4.4 Qualitative Determination — For preparing sample extract, uncap the vial containing dry sample extract. Add to it 500 µl of chloroform, and reseal with polyethylene stopper. Puncture polyethylene stopper to accommodate the needle of 10 µl syringe. Spot as rapidly as possible in subdued incandescent light 2, 5 and 10 µl on imaginary line 4 cm from bottom edge of thin-layer chromatography plate. In the presence of large quantities of extraneous material, 10 µl of the extract may not be used. Reserve the vial for quantitative analysis. On the same plate, spot 1, 3 and 5 µl standard aflatoxins solution. Also spot 5 µl of standard solution containing aflatoxins B_1, B_2, G_1 and G_2 to show that the four aflatoxins are properly resolved under conditions used. Draw line across the plate 2 to 3 cm from top edge as stop for solvent front. Also draw lines 0·5 cm from each side edge. Immediately insert plate into unlined and unequilibrated tank containing acetone/chloroform (1/9, *v/v*) as the developing system. (Position the plate to expose coated surface to maximum tank volume and seal.) Withdraw the plate from tank when solvent front reaches stop line, 12 to 14 cm above origin. Let solvent evaporate and illuminate plate from below by placing it flat, coated side up, on longwave ultraviolet lamp in darkened room. Alternatively, view plate in chromato-view cabinet or illuminated from above. (If

illumination requires looking directly at lamps, protect eyes with ultraviolet absorbing filter, such as Eastman 2A filter.) Observe pattern of 4 fluorescent spots of qualitative standard. In the order of decreasing Rf these are aflatoxins B_1, B_2, G_1 and G_2. Note small colour difference, bluish fluorescence of 'B' contrasted with slightly green 'G' aflatoxins. Examine patterns from sample for fluorescent spots having identical Rf and similar appearances to those of standards. From this preliminary plate, estimate suitable dilution for quantitative thin layer chromatography analysis. Take into account a quantity of the extract used for preliminary thin layer chromatography in final calculations.

NOTE — If four clearly identifiable spots are not visible in the qualitative standard, repeat chromatography, correcting or adjusting conditions to obtain proper resolution.

J-4.5 Quantitative Determination — Spot successively 3·5-, 5-, and two 6·5-μl portions of sample extract. All spots should be of the same size and shall not be more than 0·5 cm in diameter. On the same plate spot 3·5, 5, 6·5 μl of B_1 and G_1 standards. As internal standard spot 5 μl of B_1 and G_1 on top of one of the two 6·5 μl portions of sample. Also spot 5 μl of B_1, B_2, G_1 and G_2 as qualitative standard. Proceed as given under qualitative analysis (*see* **J-4.4**).

J-5. CALCULATIONS

J-5.1 Interpretation of the Chromatogram — Examine the pattern from the sample spot containing internal standard for aflatoxin B_1 and G_1 spots. Rf values of B_1 and G_1 used as internal standard should be the same as, or may differ only very slightly from those of respective standard aflatoxin spots. (Since spots from sample extract are compared directly with standard aflatoxin on the same plate, magnitudes of Rf are unimportant. These vary from plate to plate.) Compare sample patterns with that containing internal standard. Fluorescent spots in sample thought to be B_1 or G_1 should have Rf values identical to end colour and similar to be B_1 or G_1 used as internal standard. Identify unknown spot of sample as B_1 or G_1 only when unknown spot and internal standard spots are superimposed. Spot from sample and internal standard combined should be more intense than either sample or standard alone. Compare sample pattern with qualitative standard to determine if B_2 and G_2 are present. Compare fluorescent intensities of B_1 spot of the sample with those of the standard spots and determine which of the sample portions matches one of the standard. Ultraviolet light may be attenuated by moving the plate away from lamp so that any particular pair of spots may be compared at extinction. Interpolate, if sample spot intensity is found to be between those of two standard spots. If spots of smallest portion of sample are too intense to match standards, dilute sample and rechromatograph. Compare G_1 spots in the same manner. Assume that B_1 and B_2 have same fluorescent intensity to mass relationships and compare B_2 spots of sample with B_1 standard spots to make quantitative

estimate of B_2. Likewise, assume G_2 has the same fluorescent intensity to mass relationship as G_1 and compare G_2 spot of the sample with G_1 spot of the standard.

J-5.1.1 *Calculation* — Calculate aflatoxin from the following formula:

$$\text{Aflatoxin } B_1 \text{ in } \mu g/kg = S \times \Upsilon \times \frac{V}{X} \times M$$

where

S = µl of aflatoxin B_1 standard equal to unknown;

Υ = concentration of aflatoxin B_1 standard µg/ml;

V = volume in µl of final dilution of sample extract;

X = µl of sample extract spotted giving fluorescent intensity equal to S, the aflatoxin B_1 standard; and

M = mass in g of sample applied to silica column.

NOTE — For 10 g of sample extract 50 ml of aqueous methyl alcohol extract is used. If final extract dilution does not represent 10 g, calculate correct sample mass and substitute.

J-5.1.2 Calculate aflatoxin G_1 as given above for aflatoxin B_1.

J-5.1.3 Calculate aflatoxin B_2 as ' µg aflatoxin B_2 per kilogram based on fluorescence of aflatoxin B_1 '.

J-5.1.4 Calculate aflatoxin G_2 as ' µg aflatoxin G_2 per kilogram based on fluorescence of aflatoxin G_1'.

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